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Stabilizing Protein Effects on the Pressure Sensitivity of Fluorescent Gold Nanoclusters

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14. ABSTRACT
This report describes the effect of scaffold protein secondary structure on the pressure response of protein-stabilized gold nanoclusters (P:NCs). These studies were conducted using an ethanol-driven pressure cell incorporated into a spectrofluorometer to measure the pressure sensitivity of P-NCs in real time. Results demonstrate that the pressure response of P:NCs is indeed dependent on the secondary structure of the protein. Proteins with high beta sheet content such as pepsin and DNase do not show a pressure response in the range studied (0–400 MPa), whereas fully alpha helical proteins like bovine serum albumin show linear pressure response over the full range. Interestingly, proteins low in secondary structure and high in random coil (i.e., lysozyme, 60% coil) show linear pressure response at pressures ranging up to 200 MPa but no response at higher pressures.

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1. Introduction and Background

Protein-stabilized nanoclusters (P:NCs) were first synthesized in 2009 using gold (Au) ions and bovine serum albumin (BSA) as both the stabilizing and reducing agent.[1] Since then P:NCs have been synthesized using several different proteins with varied structure and function.[2] These proteins range from the fully alpha helical serum protein BSA[3–7] to the enzymes such as lysozyme[8,9] and DNase 1,[10] which are composed mostly of random loops and coils and high beta sheet content, respectively. In recent years P:NCs have been shown to have an increased level of tailorability through the use of different metals such as silver, copper, or platinum and by controlling the cluster size though reaction conditions.[4,11–14]

An interesting attribute of P:NCs is that NC synthesis does not affect protein function, but the fluorescence of the P:NCs is largely affected by the environment of the stabilizing protein, allowing these hybrid systems to act as sensors in many applications.[2,9,14–19] This has led to the creation of a multitude of P:NC-based sensors that use the chemical interactions of the stabilizing protein and its environment to detect the presence and concentration of a wide variety of substances including heavy metals and biological molecules.[19] However, although a plethora of applications have been created to use the functional behavior of the stabilizing proteins of P:NCs to monitor chemical properties of the environment, there has been very limited research into using P:NCs to monitor mechanical/structural effects such as pressure response.

In 2013, Zhang et al.[20] conducted the first study on the response of P:NCs to mechanical forces by studying the pressure response of BSA:Au nanoclusters (AuNCs) inside a diamond anvil cell. BSA:AuNCs exhibited a linear increase in fluorescence intensity proportional to the pressure increase in the gigapascal (GPa) regime. More recently, our group conducted a study of BSA:AuNCs using a fluorimeter-based ethanol-driven pressure cell to study lower pressures from 0 to 400 MPa.[21] However, these results are limited to the behavior of BSA:AuNCs and do not address if this is a widespread phenomenon among P:NCs or if the observed pressure response is limited to BSA-stabilized clusters. BSA is a unique protein; it fully comprises alpha helices, or springs. Thus, the observed pressure response is not surprising, as the protein is highly compressible.

In this investigation the effect of secondary structure composition on pressure response of red-emitting P:AuNCs was studied using 4 proteins of varied secondary structure elements (see Table 1 and Fig. 1). Results demonstrate that the pressure response of P:NCs is indeed dependent on the secondary structure of the protein. Proteins with high beta sheet content such as pepsin and DNase do not show a

pressure response in the range studied (0–400 MPa), whereas fully alpha helical proteins like BSA show linear pressure response over the full range. Interestingly, proteins low in secondary structure and high in random coil (i.e., lysozyme, 60% coil) show linear pressure response at pressures ranging up to 200 MPa but no response at higher pressures.

Table 1 Secondary structure content of the 4 proteins studied

Protein	α-helix (%)	B-sheet (%)	Turn (%)	Extended chain (%)
BSA	67	0	10	23
Lysozyme	30	13	27	30
DNase	40	34	12	12
Pepsin	3.4	63.1	7.3	26.2

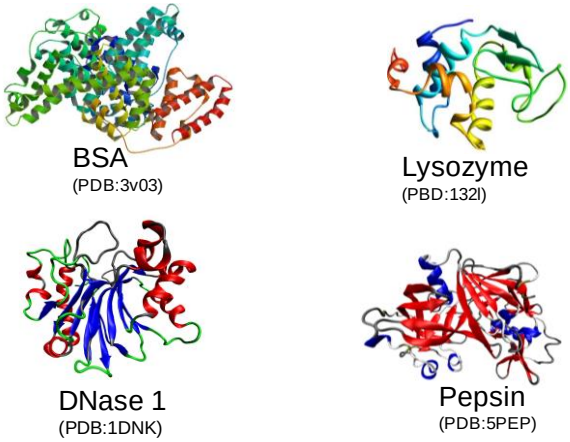

Fig. 1 Crystal structures of proteins used in this study

2. Synthesis of Materials

2.1 Synthesis of BSA-Stabilized Gold Nanoclusters

A solution of BSA:AuNCs was synthesized using published methods.[1] Aqueous chloroauric acid ($HAuCl_4$) solution (5 mL, 10 mM) was added to BSA solution (5 mL, 50 mg/mL) and stirred vigorously for 2 min. Sodium hydroxide (NaOH) solution (0.5 mL, 1 M) was subsequently added to bring the pH of the solution to 12. The solution was then incubated at 37 °C overnight while stirring until the solution turned a dark brown color. The BSA:AuNCs solution was then spin-purified using a 5-kDa MWCO spin filter several times to remove excess Au salt. The purified sample was lyophilized and resuspended at a concentration of 10 mg/mL in ultrapure water.

2.2 Synthesis of Lysozyme-Stabilized Gold Nanoclusters

A solution of lysozyme:AuNCs was synthesized using published methods.[9] Aqueous $HAuCl_4$ solution (5 mL, 5 mM) and lysozyme (5 mL, 10 mg/mL) were added to 5 mL of ultrapure water and stirred vigorously for 5 min. NaOH solution (0.5 mL, 1 M) was subsequently added to bring the pH to 12 and, the solution was incubated at 37 °C overnight while stirring. The solution was then spin-purified using a 5-kDa MWCO spin filter several times to remove excess Au salt. The purified sample was lyophilized and resuspended at a concentration of 10 mg/mL in ultrapure water.

2.3 Synthesis of Pepsin-Stabilized Gold Nanoclusters

A solution of pepsin:AuNCs was synthesized using published methods.[22] Aqueous $HAuCl_4$ solution (5 mL, 5 mM) was added to pepsin solution (5 mL, 40 mg/mL) while stirring vigorously. NaOH solution (1.5 mL, 1 M) was subsequently added to bring the pH to 12, and the solution was incubated at 37 °C while stirring for 4 h. The solution was then spin-purified using a 5-kDa MWCO spin filter several times to remove excess Au salt. The purified sample was lyophilized and resuspended at a concentration of 10 mg/mL in ultrapure water.

2.4 Synthesis of DNase-Stabilized Gold Nanoclusters

A solution of DNase:AuNCs was synthesized using published methods.[10] Aqueous $HAuCl_4$ solution (5 mL, 5 mM) was added to DNase 1 solution (5 mL, 20 mg/mL) while stirring vigorously. NaOH solution (0.5 mL, 1 M) was subsequently added to bring the pH to 12, and the solution was incubated at 37 °C while stirring for 12 h. The solution was then spin-purified using a 5 kDa MWCO spin filter several times to remove excess Au salt. The purified sample was lyophilized and resuspended at a concentration of 10 mg/mL in ultrapure water.

2.5 Ethanol-Driven Hydrostatic Pressure Cell

See *Use of an Ethanol-Driven Pressure Cell to Measure Hydrostatic Pressure Response of Protein-Stabilized Gold Nanoclusters*[21] for complete instructions for the operation of the ethanol-driven hydrostatic pressure cell. The P:NC solutions were each resuspended in ultrapure water at a concentration of 10 mg/mL for hydrostatic pressure response analysis.

3. Results and Discussion

P:NCs with bright red fluorescence were successfully synthesized using BSA, lysozyme, DNase 1, and pepsin (see Figs. 2A and 2B). The measured emission spectra show peaks ranging from 630 to 650 nm for all P:NCs. These spectra are consistent with the fluorescence spectra of 25-atom P:NCs found in literature.[1,9,10,17,21]

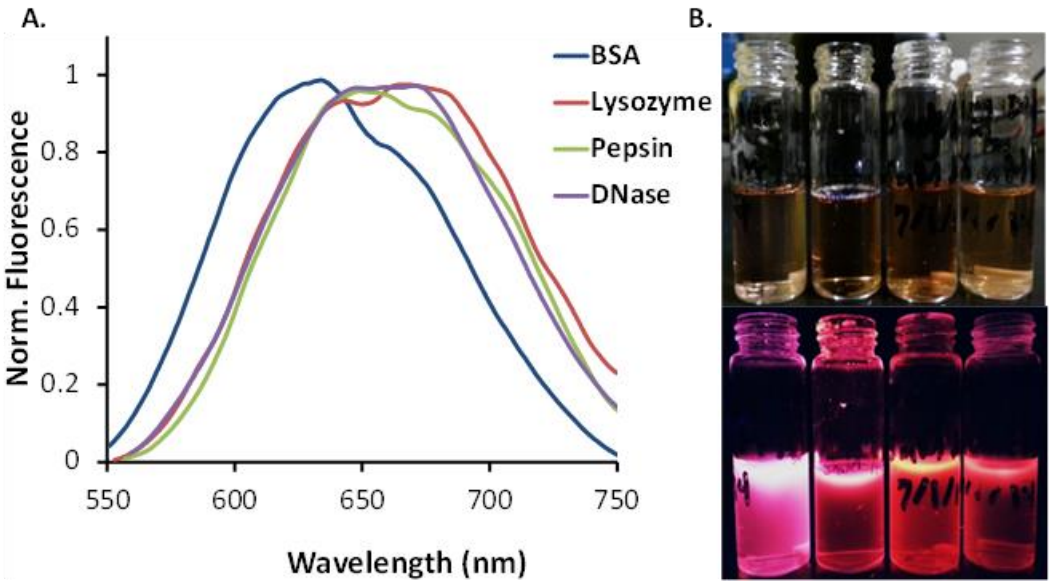

Fig. 2 A) Fluorescence emission spectra of P:NCs upon excitation at 488 nm and B) P:NCs under brightfield illumination (top) and UV illumination (bottom); (left to right) BSA, lysozyme, DNase 1, and pepsin AuNCs

The pressure dependence of BSA:, lysozyme:, DNase 1:, and pepsin:AuNC fluorescence was characterized using an ethanol-driven pressure cell from 0 to 400 MPa. The effect of secondary structure on pressure response was then characterized for the 4 different P:NCs. These results are summarized in Fig. 3.

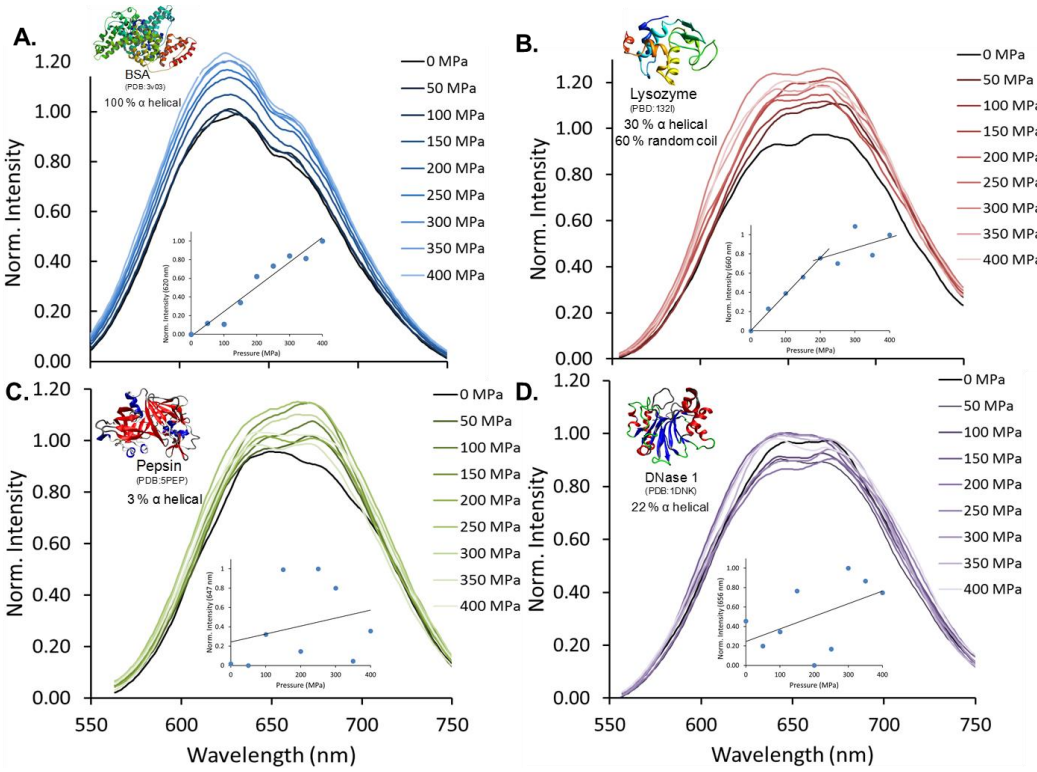

Fig. 3 Pressure response of P:NCs tested: A) fully alpha helical BSA:AuNCs, B) random-coil-dominated lysozyme:AuNCs, C) beta-sheet-dominated pepsin:AuNCs, and D) beta-sheet-dominated DNase:AuNCs

BSA- and lysozyme-stabilized AuNCs exhibit linear pressure-induced fluorescence enhancement, whereas pepsin- and DNase-stabilized AuNCs do not exhibit any pressure response in the investigated pressure range. Interestingly, while BSA:AuNCs maintain a linear pressure response over the full range studied (0–400 MPa), lysozyme:AuNCs exhibit a dramatic decrease in pressure response at pressures higher than 150 MPa.

Although the exact mechanisms of this change in pressure response are currently unknown; the flexible element (alpha helix, extended coil and turn) content of the stabilizing proteins could play a major role. Table 1 highlights the much higher flexible element content of BSA and lysozyme—100% and 87%, respectively—than DNase (64%) and pepsin (37%), which may explain why the latter 2 P:NCs show a negligible pressure response.

Understanding the role secondary structure plays in pressure response may be simplified if the secondary structure elements are thought of as common objects. Alpha helices can be thought of as highly compressible springs, extended chains and turns are like floppy strings, and beta sheets are like inflexible boards. The pressure response observed for BSA:AuNCs was hypothesized to be due to the

compression of protein ligands around the AuNC, thus leading to enhanced electronic density and higher fluorescence. In our study, the intensity of the fluorescence peak of the BSA:AuNCs exhibits a significant linear relationship with applied pressure. This results in an increase in fluorescence intensity of approximately 23% at 400 MPa. BSA is composed almost entirely of alpha helices. It is logical that the protein would show a linear response over a wide pressure range (0–2 GPa, combining our study and the study conducted by Zhang et al.[20]), as the protein's spring-like structure would be able to compress quickly and continuously over a wide range of pressure.

Interestingly, the percentage of turn and extended chain structural components for lysozyme comprises more than half (57%) of the secondary structure elements of the protein. The higher extended chain content found in lysozyme may be attributed to the steep pressure dependence observed from 0 to 150 MPa, as, stated vide supra, the extended coil and turn elements are similar to strings in that they are very quickly and easily compressed to their noncompressible/final state. The fact that lysozyme has nearly twice the random coil content as BSA correlates with the pressure response of the lysozyme:AuNCs under 150 MPa, which is approximately twice as high as that of the BSA:AuNCs, at 0.1%/MPa versus 0.06%/MPa, respectively, as shown in Table 2. As the random coil structure is more mechanically compliant than the α-helical and β-sheet structures, one might hypothesize that they would dominate the low-pressure response of the P:NCs.

Table 2 Pressure response for the various P:AuNCs tested in this study

Protein	Pressure response (%/MPa)	R^2
BSA	0.06	0.94
Lysozyme <150 MPa	0.10	0.94
Lysozyme >150 MPa	0.04	0.36
Pepsin	0.02	0.28
DNase	0.01	0.082

Note: R^2 = coefficient of determination.

Looking at pressures above 150 MPa, it can be seen that a dramatic decrease occurs in the pressure response of the lysozyme:AuNCs. In this high-pressure region, the BSA:AuNCs maintain a linear pressure response of 0.06%/MPa while the lysozyme:AuNCs exhibit a significant drop in pressure response to 0.04%/MPa. Again, the exact mechanisms of this behavior are unknown and will require further study. However, it can be seen in Table 1 that this behavior seems to correlate with the percentage of α-helical structure in the protein. BSA, which has a very high percentage of α-helical structure, exhibits a much higher pressure response in this region than the lysozyme:AuNCs. The pepsin:AuNCs, which have virtually no α-helical structure, exhibit a negligible pressure response in this region. The fact

that the mixed α-β DNase:AuNCs do not exhibit pressure response in this region despite having a significant percentage of α-helical structure suggests that the pressure response in this pressure region is a function of the ratio of α-helix and β-sheet structure present in the stabilizing protein.

These data support the hypothesis that the pressure-induced fluorescence enhancement experienced by P:NCs is influenced by the mechanical structure of the stabilizing protein. The high random coil content of lysozyme gives it a much more compliant structure than BSA. This would explain the very high response of lysozyme:AuNCs compared with the BSA:AuNCs. The drop in pressure response of the lysozyme:AuNCs at pressures above 150 MPa can likely be attributed to the complete collapse of the lysozyme structure at very high pressures, as opposed to the stiffer BSA structure, which can maintain its linear pressure response at these higher pressures.

4. Summary and Conclusions

In this study, the influence of the secondary structure of protein-stabilized nanoclusters, or P:NCs, on the pressure-induced fluorescence enhancement was investigated. Red-emitting AuNCs composed of 25 atoms were synthesized using a variety of stabilizing proteins with various secondary structural properties and subjected to increasing hydrostatic pressures in the range of 0–400 MPa using an ethanol-driven pressure cell. The influence of the pressure on the fluorescence emission peak was measured using a spectrofluorometer at 50-MPa intervals.

It was found that BSA- and lysozyme-stabilized AuNCs exhibit pressure-induced fluorescence enhancement in the range of 0–400 MPa while DNase- and pepsin-stabilized AuNCs exhibit a negligible pressure response. It was also found that the lysozyme:AuNCs showed a significant difference in pressure response when comparing pressures lower and higher than 150 MPa.

These data demonstrated that the secondary structure of the stabilizing protein has a major effect on the pressure-induced fluorescence enhancement of the P:NCs. The results indicate that at pressures lower than 150 MPa the random coil component of the protein structure dominates the pressure response, while at pressures higher than 150 MPa the pressure response is dominated by a function of the α-helix and β-sheet structure, with a higher-percentage α-helical structure yielding a higher-pressure response.

The results found in this study have helped illuminate the effect of protein structure on the pressure response of P:NCs. However, further testing will need to be completed to get a full understanding of the effect of protein structure on pressure-induced fluorescence enhancement. This testing will include examining the fluorescence response of a wider variety of protein structures as well as examining the changes to the protein structure directly as a response to pressure using Fourier transform infrared approaches used in previous work.[20]

Once the effects of protein structure on the pressure response of P:NCs is understood, investigations into the effect of various metals and nanocluster sizes can be initiated. This information will allow for a high degree of control over the pressure response of synthesized P:NCs, resulting in a suite of potential pressure monitoring devices for various pressure applications.

Future work will also include an investigation into the effect of embedding the pressure-sensitive P:NCs into a clear polymer matrix. Although it has been determined that P:NCs retain their fluorescent properties when embedded in a polymer matrix (data not shown), the pressure response of these embedded P:NCs has yet to be investigated.

The results of this preliminary investigation are very promising and show that not only do P:NCs exhibit a linear pressure-induced fluorescence enhancement, but that this pressure response can be tuned by varying the secondary structure of the stabilizing protein.

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List of Symbols, Abbreviations, and Acronyms

Au	gold
AuNC	BSA:Au nanocluster
BSA	bovine serum albumin
GPa	gigapascal
$HAuCl_4$	chloroauric acid
NaOH	sodium hydroxide
P:NC	protein-stabilized nanocluster

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